2

SEROTONIN *N*-ACETYLTRANSFERASE

A Personal Historical Perspective

D. C. Klein

Section on Neuroendocrinology
Laboratory of Developmental Neurobiology
National Institute of Child Health and Development
National Institutes of Health
Bethesda, Maryland

This chapter is written as part of a 40 year celebration of the discovery of melatonin and it seems most appropriate to this invited author to contribute something of a personal historical nature. Accordingly, the subject matter of the chapter is my relationship with serotonin *N*-acetyltransferase and the people who participated in the major advances in understanding this protein. Readers wanting a detailed up-to-date 1998 accounting of what's known about the enzyme are directed to other publications (1–11); earlier reviews will also be of interest (12–16).

1. THE PINEAL GLAND: A MELATONIN FACTORY ON A ~ 24-HOUR SCHEDULE

At the time I started working in this field around 1968, Aaron Lerner's discoveries of melatonin and the high concentrations of melatonin in the pineal gland were well known (17,18), as was the high amount of serotonin in the pineal gland (19). Julie Axelrod and his coworkers were generally interested in biogenic amine metabolizing enzymes, so it was not long before Lerner's findings led to their demonstration that the pineal gland had the enzymatic machinery required to convert serotonin to N-acetylserotonin and N-acetylserotonin to melatonin (20,21). This established the pineal gland as a melatonin factory.

The concept that there are 24-hour rhythmic changes in pineal indoles came from studies by Quay (22,23). He had developed fluorescent techniques to measure pineal melatonin and reported that there was a large day/night rhythm in serotonin and

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melatonin. The large circadian changes in these compounds were intriguing and immediately raised questions in the minds of curious investigators about how changes in the concentrations of biogenic amines in neural tissues are regulated.

Interest in the regulation of melatonin levels first focused on the last enzyme in melatonin synthesis, hydroxyindole-O-methyltransferase (HIOMT). Axelrod and his coworkers characterized the protein and found that high levels were found only in the pineal gland, based on studies in mammals (12,24–27). In contrast, serotonin acetylation was thought to be mediated by a widely distributed enzyme, and therefore was not viewed as being a likely site of regulation (12). This acetyltransferase, arylamine *N*-acetyltransferase (E.C. 2.3.1.5), played an essential role in the discovery of acetyl CoA (28), and was well known at the time. Faced with the alternatives of either HIOMT or arylalkylamine N-acetyltransferase as the key regulator of melatonin production, Axelrod's team focused on the former—acetylation was ignored.

This hunch looked pretty good at first, because a positive relationship seemed to exist between environmental lighting, HIOMT and melatonin: HIOMT activity enzyme decreased following long-term exposure to light and decreased following long periods in continual lighting (12,25–27). In addition, melatonin was known to be high in the dark of night and low during the day, which matched up with the report that there was a large daily rhythm in HIOMT activity. Based on this, it seemed reasonable that HIOMT regulated the rhythm in melatonin production.

Over the years, the finding that HIOMT activity is elevated in the rat pineal gland following long term exposure to darkness and lower following light has been confirmed (29). However, the claim regarding HIOMT as a regulator of the melatonin rhythm didn't fare so well.

2. THE ROLE OF N-ACETYLTRANSFERASE IN REGULATING RHYTHMS IN PINEAL INDOLE METABOLISM

My entre into the pineal field came after hearing Russell Reiter give a seminar entitled "The Truth About the Pineal Gland", which I attended while both of us were at the University of Rochester. I came there in 1967 from Rice University after receiving my Ph.D. on the recently discovered thyroid hormone calcitonin. My goal in Rochester was to use *in vitro* techniques to study the hormonal control of bone metabolism as a fellow with Larry Raisz. After about a year of bone work, I realized that if I was going to make a contribution to science and establish a productive independent research program, the chances were slim that this could be done in the bone field. This area was filled with many well established, large and intensely competitive groups. I started to look around for new fields to conquer. I liked endocrinology and a look at my endocrinology text gave me my some indication of where I should go: the pineal gland or thymus. The reason? Not too much seemed to have been known about these glands since their chapters were the shortest in the book. Reiter's talk then helped turn me towards the pineal gland.

I learned more about melatonin primarily from articles by Axelrod's group, especially those that were coauthored by Dick Wurtman (12,25–27). These convinced me that there were lots of interesting open questions in the area. With Raisz's generous support and encouragement, I started to study melatonin.

The first step was to establish a pineal organ culture system, which proved to be easy—I simply replaced the bones with pineal glands. This worked and the glands

seemed quite happy. They made melatonin from radiolabeled precursors and responded to norepinephrine (13,30), as had been reported by Axelrod and Wurtman, working with Harvey Schein (31). The melatonin response was robust, about 10-fold. My initial goal was to demonstrate that this was accompanied by an increase in HIOMT activity—which would confirm the then popular HIOMT hypothesis. However, norepinephrine treatment didn't increase HIOMT activity (13). Similarly, I discovered that dibutyryl cyclic AMP elevated melatonin production without increasing HIOMT activity (32), which provided more reason to question the role of HIOMT in regulating melatonin production.

The frustration with the *in vitro* results with HIOMT led me to reexamine the *in vivo* HIOMT rhythm. Again I was frustrated because I was unable to confirm the large nocturnal increase in activity that had been reported (21,25–27). I optimized the assay, following the directions of Angelo Notides, an excellent biochemist in the department, and also consulted with Axelrod and Wurtman. I tried everything. Still no luck—I was only able to find very small day/night difference at some ages (33), but was never able to find the large day/night rhythm that was in the literature.

I was faced with a puzzle. If HIOMT did not regulate the large change in melatonin production, what did? To get a better picture of what was taking place in the synthesis of melatonin I turned to Notides again for help and we developed a thin layer chromatography (TLC) system which separated serotonin oxidation and N-acetylation products (34). This was used to study effects of compounds which elevate melatonin production. The list now included the monoamine oxidase inhibitor harmine, in addition to norepinephrine and dibutyryl cyclic AMP (13,30,35). Glands were incubated with radiolabeled tryptophan or serotonin and a sample of gland homogenate or media extract was mixed with authentic standards and the mixture was resolved by TLC. In all cases the results were unequivocal—whenever there was an increase in the accumulation of radiolabeled melatonin production, the accumulation of radiolabeled N-acetylserotonin increased. I also found it was possible to increase melatonin production simply by elevating *N*-acetylserotonin. Based on these findings I suspected that the rate of melatonin production could increase simply by increasing the amount of *N*-acetylserotonin; and, that compounds which elevate melatonin production may do this by elevating the *N*-acetylation of serotonin.

In July of 1969 I moved to the National Institute of Child Health and Human Development (NICHD) of the National Institutes of Health (NIH). I had learned about the opportunity from Axelrod and was thrilled with the opportunity to work independently at the NIH.

I was given a tiny laboratory located in the basement of the living quarters of the women's branch of the U.S. Navy—the WAVES' Barracks. This was located in the National Naval Medical Center, which is separated from the NIH by a main thoroughfare. The labs were windowless (good for pineal work) and often flooded with heavy rains (bad for feet). These minor problems were easily ignored because waiting for me when I took the new job was a very nice surprise, an unusually capable and dedicated person, who shared my enthusiasm for pineal work, penchant for hard work and concern for detail—Joan Weller.

The pineal organ culture system was quickly reestablished and we started to work with it; we also tried to set up an assay to measure pineal serotonin N-acetyltransferase activity. This proved to be very difficult because we used daytime glands, which,—as we now know—have nearly undetectable levels of serotonin *N*-acetyltransferase activity. Hence, we were forced by circumstance to invent a very sensitive and specific assay

so that we could selectively and reliably detect very low levels of serotonin *N*-acetyltransferase activity. The key element was to use TLC (34) to isolate products.

The first breakthrough with this assay came from the pineal organ culture system: treatment with norepinephrine or dibutyryl cyclic AMP treatment increased serotonin *N*-acetyltransferase activity (30,36). We also found that this was blocked by cycloheximide. I recall that the first results came on Thanksgiving Day.

The effects of norepinephrine or dibutyryl cyclic AMP on *N*-acetyltransferase activity were small. In 20–20 hindsight it is clear that our treatment period was not optimal—it was too long, allowing stimulated values to fall towards control levels. However, the response was sufficient to establish the fundamental principle that these compounds could elevate serotonin N-acetyltransferase activity and that this explained how they elevated melatonin production (30). Although these *in vitro* studies were important, they didn't provide enough evidence to prove what was happening *in vivo*. It was certainly possible that these *in vitro* results were not physiologically relevant.

I turned to *in vivo* studies and first tried to elevate serotonin *N*-acetyltransferase activity by keeping animals in the dark. This reasoning was based in part on the HIOMT studies. I was unfamiliar with the concepts of an endogenous clock and circadian rhythms, and only obtained tissue during normal working hours. I didn't know then that clock control of the enzyme would prevent an increase during the day. Soon, however, the concept of an endogenous clock would dawn on me.

The next big breakthrough came when I started to obtain tissue at night. There wasn't a proper animal room in the basement of the WAVES' Barracks, so I had to fix up a small closet with shelves for cages. I light-proofed the door and used a timer to control lighting. To obtain tissue, animals were taken down the hall to the laboratory and pineal glands were removed and placed in individual micro tubes—all this under room light.

When the glands were assayed, I was surprised and elated to see numbers I had never dreamed of seeing! Whereas I had become accustom to scintillation counter read-outs of 100 or 200 DPM, I was now seeing some counts in the tens of thousands!!! I was still troubled, however, because there was enormous variation—some tubes had counts in the hundreds, some in the thousands and some in the tens of thousands.

Why the large variation? Two minor details were overlooked—the tubes were not numbered and I didn't record when animals were removed from the holding space and when the glands were put on dry ice. As a result, I had no way of determining if there was a correlation between enzyme activity and time in light. When I repeated the experiments I did a better job of record keeping and it became apparent that the highest DPMs came from animals that were in light for the least amount of time.

When I put this data together with daytime data, it was clear that I had discovered that *N*-acetyltransferase activity exhibited a very large day/night rhythm and that light exposure at night causes enzyme activity to drop very rapidly. So, in one single experiment I had the principles of two Science papers!! One reporting the day/night rhythm in serotonin N-acetyltransferase (37) and the second reporting light-induced rapid turn-off (38).

I was overwhelmed with excitement about the discoveries. I started to obtain night tissue without exposing animals to room light at all, by using dim red light. Soon I was able to demonstrate that the rhythm persisted in constant darkness, indicating it was driven by an endogenous clock (37).

The first publication of the rhythm received a lot of attention, because it redirected thinking in the field by establishing that serotonin *N*-acetyltransferase was the

key regulator of the circadian rhythm in pineal melatonin and that the reciprocal changes in serotonin and melatonin were due to changes in the activity of serotonin N-acetyltransferase. It was proposed that serotonin N-acetylation limited the amount of melatonin made during the day; and, that at night melatonin production was increased by the increase in *N*-acetylserotonin which acted *via* mass action. The report also indicated that at night melatonin may be limited by HIOMT activity. This encompassing concept is valid for all vertebrates and has become a cornerstone of pineal biochemistry. For this reason the paper was identified as a Citation Classic and is one of the highest cited papers in the pineal and circadian literature (39). It is of incidental interest that another 1970 paper of mine, one on bone metabolism with Raisz, also became a Citation Classic (40). *What a great year!!!*

Although I never worked with Axelrod on the pineal gland, I consulted with him regularly about my work when I was at the NIH. At the time, no one in his laboratory was working on the pineal gland. Axelrod reviewed my manuscripts and showed me how to state things simply in easily understood language. The dramatic results he was reading about in my papers made him itchy to get back into the pineal field and as a result he eventually was part of highly productive work done with a series of coworkers, including Takeo Deguchi and Marty Zatz.

Although I quickly published the work on the acetyltransferase rhythm, the data on the rapid effect of light on serotonin N-acetyltransferase activity sat around for a while. I didn't realize how important it was until I looked at it side-by-side with a report from Helena Illnerová indicating that light exposure at night causes a very rapid increase in serotonin (41), which is otherwise very low at night. The rapid effect inhibitory effect of light on acetylation seemed to explain this. Photic suppression of N-acetyltransferase activity was of broad interest because of the rapidity of the effect, which suggested that remarkable regulatory mechanisms exist to trigger this. I wrote to Illnerová about my idea that the rapid light-induced increase in serotonin was due to the decrease in serotonin acetylation. She was in full agreement with my thinking and subsequently published extensively using serotonin N-acetyltransferase as an output marker for the suprachiasmatic nucleus (SCN) (42). I didn't meet Illnerová for several years. However, we finally met at a meeting on stress in Czechoslovakia in 1975. This meeting firmed up our scientific relationship and started a long friendship. It also was an important step towards establishing mechanisms through which Illnerová and one of her students, Jiri Vanecek, could come to the U.S. to work with me.

From the work done very early in the 70's it was clear that regulation of serotonin N-acetyltransferase was complex and important; and, that it begged for a molecular investigation. Although insufficient material was available for this type of work, it was possible to use the rhythm in serotonin N-acetyltransferase for other purposes. It played an important role as an output marker to describe the neural circuit which regulates the pineal gland (43). Of special note is the body of work which identified the SCN as the source of signals which control circadian rhythms in the pineal gland (44,45). This was accomplished in a collaborative study involving Bob Moore. This advance proved to be important on a broader scale because it helped establish the SCN as the "The Mind's Clock" (46).

Other important developments reflected some interesting pharmacological and biochemical observations. One came from Andy Parfitt, who found that depolarizing agents block adrenergic-cyclic AMP induction of serotonin N-acetyltransferase activity (47). David Sugden and I figured out that distinct β- and α-adrenergic components regulate the enzyme (48). "Namboo" M.A.A. Namboodiri established that serotonin

N-acetyltransferase was inactivated by protein thiol-disulfide exchange (49–51). And, the late Michel Buda found that a drop in cyclic AMP was the signal for the rapid decrease in enzyme activity (52).

Important advances were also made on the characterization of the enzyme. This started with communications with Wendell Weber, an expert on liver arylamine N-acetyltransferase. Both of us suspected the pineal enzyme with the rhythm in activity may not be arylamine N-acetyltransferase. We exchanged tissues and discovered that pineal homogenates which exhibited large differences in serotonin acetylation did not exhibit a day/night difference in arylamine N-acetyltransferase activity.

This convinced me that a detailed biochemical analysis of amine acetylation by the pineal gland might result in the characterization of a novel enzyme that was dedicated to melatonin synthesis. The effort was undertaken by Pierre Voisin and Namboodiri using both rat and sheep pineal glands (53). The work clearly established that the enzyme which exhibited the large day/night differences in serotonin acetylation was not arylamine N-acetyltransferase (EC 2.3.1.5), and that an arylalkylamine *N*-acetyltransferase (AANAT) could be chromatographically resolved from arylamine N-acetyltransferase in pineal homogenates. The enzymes also exhibited distinctly different biochemical characteristics. AANAT was given the designation EC 2.3.1.87.

3. CLONING OF AANAT

Sometime after the identification of AANAT as a novel enzyme, it became apparent that the developing cloning technology would eventually allow us to obtain large amounts of AANAT and the tools necessary to study how it is regulated. At the start of this period, techniques were primitive and inefficient; certainly, molecular biology kits, reagents and commercial services were a thing of dreams. However, I was confident that sooner or later everything would fall into place and techniques required to clone AANAT would be available. I started to position ourselves accordingly by trying to do as much pineal-related molecular biology as possible. Joan Weller and I started with efforts aimed at purifying intact mRNA and making pineal cDNA libraries.

I also tried to identify relevant goals that would help us learn how to use molecular techniques. One of our first efforts was aimed at cloning an interesting protein found in both the pineal and retina—which was termed S-antigen at the time and now is called arrestin. I suspected that this could be cloned using an expression strategy in which protein was detected using antisera. At the time I was using Igal Gery's anti-S-antigen sera for other purposes and thought it might be used to clone S-antigen. I turned to Toshi Shinohara for help, because he had made and was using a bovine retinal cDNA library. We discussed cloning S-antigen and I brought him one of my postdoctoral fellows who wanted to learn molecular biology—Cheryl Craft. Bovine S-antigen was cloned shortly thereafter (54).

After getting our feet wet with the S-antigen project, we turned our attention to cloning other proteins of interest, including human HIOMT. This was done using a homology approach with probes based on the bovine sequence published by Takeo Deguchi's group (55). The first steps towards cloning human HIOMT were made by Joan Weller and Helena Illnerova; the work was completed by Susan Donohue who received help from Pat Roseboom (56). This was done entirely within our group and represented an important training experience.

Still looming over our program was the goal of cloning serotonin N-acetyltransferase. Several approaches had been tried over the years, all without success. Things started to happen in 1994. At that time the molecular biology experience and expertise in the laboratory was high. The program included Ruben Baler, who brought a sharp and nimble mind and excellent technical knowledge. Baler had just finished a challenging project on the identification and regulation of pineal Fos related protein-2 and was in full stride (57). Pat Roseboom had gained significant hands-on experience by cloning two interesting proteins, 14-3-3 ε and ζ (58), which in fact represented the unintended outcome of a failed attempt to clone AANAT.

Other members of the team were Steve Coon, who joined the program because of a common interest in adrenergic receptors and his desire to learn molecular biology; and, Marianne Bernard, who was strongly focused on the molecular regulation of HIOMT. The group was formidable in both expertise and intellectually combativeness. I convinced nearly everyone that it would be to each person's benefit if AANAT were to be cloned and it was agreed that other projects would be given lower priority, so that all possible approaches to cloning AANAT could be tried or retried.

The method that worked was one in which we had the least amount of confidence—an expression cloning strategy. We were encouraged by the success of Steve Reppert's program with this method, which was used to clone the first melatonin receptor (59). Immediately after hearing about this in February of 1996 at a Gordon Conference we set up the method. We thought that the enzyme was too unstable and the pineal gland was too specialized for this to occur in nonpineal cells. However, we were wrong.

The expression cloning strategy was spearheaded by Coon, who generated an ovine cDNA library and used a very sensitive AANAT activity assay that Baler and I had developed to detect expression. The method resolved products by TLC and visualized them with the PhosphoImager. Everything worked quite well and the start-to-finish time for this effort was remarkably short. Images verifying that AANAT had been cloned were faxed to me in May when I was in Italy attending a meeting. Immediately after cloning the ovine enzyme, the rat enzyme was cloned by Pat Roseboom. In July the paper describing the cloning of ovine serotonin N-acetyltransferase was submitted1. It included the findings that large rhythm in enzyme activity was accompanied by a large AANAT mRNA rhythm in the rat, but nearly no rhythm in the sheep. Analysis of the sequences indicated that AANAT and arylamine *N*-acetyltransferase were in different superfamilies—explaining why all previous efforts based on homology screening never worked.

This advance played an important role in the cloning of AANAT from other vertebrates and in the molecular analysis of the enzyme (8). Some of the most important advances made in my laboratory involved analysis of the AANAT promoter by Ruben Baler (6), which has led to rat transgenesis studies with David Carter. These have identified a sequence in the AANAT gene which directs tissue and time-of-day specific expression in transgenic rats. It might allow us to construct lines of rats that express genetic information of our choice selectively in the pineal gland.

Work by Pat Roseboom described regulation of AANAT mRNA in the rat (5) and work done by Marianne Bernard demonstrated that the expression of the AANAT gene in the chicken is regulated in a circadian manner and is driven by a pineal clock (7,56). This reflected collaborative projects done with Mike Iuvone and Marty Zatz. Work on the chicken pineal and retina is being extended by Nelson Chong and Iuvone.

The cloning of AANAT has led to a productive collaboration on the fish pineal gland with Välerie Bégay, Jack Falcón, Greg Cahill and Steve Coon (9). This established that the pineal clock drives a rhythm in AANAT mRNA in the pike and zebrafish, but that AANAT mRNA in the trout is expressed in a tonic manner. This fish effort also had the unexpected and surprising result of the identification of a second AANAT gene in pike. This may lead to identification of such a second gene in other species. Related to these studies on fish AANAT are efforts by Yoav Gothilf in my group to use AANAT as a marker gene to use to study pineal development in the zebrafish. He's detected AANAT mRNA in the embryonic pineal gland 22 hours postfertilization!

Use of the ovine AANAT sequence made it possible for Steve Coon and Ignacio Rodriguez to clone the human gene and to determine the chromosomal localization (3). This advance may provide a key step towards the identification of a molecular basis of low melatonin production in humans, as has been done by Pat Roseboom in the mouse. Roseboom found that low levels of AANAT activity occur in the C57/Bl mouse because of a mutation that causes missplicing and results in formation of a severely truncated protein (61).

One of the minor surprises which came out of the cloning of AANAT was that the gene is expressed in places and patterns that were not expected (8). Although it was expected that the retina would express the gene, Pat Roseboom discovered that rat retinal AANAT mRNA exhibits a day/night rhythm (5). And, Vince Cassone made the surprising finding that chick retina AANAT mRNA occurs in non-photoreceptor cells (7). Several efforts by Coon with others have resulted in the identification of AANAT transcripts outside the pineal gland and retina, including the pituitary gland, certain brain regions and the ovary (1–3,7,62). Others have discovered AANAT in the testis (63).

Soon after AANAT was cloned, we started to make antisera. Expressed protein and peptides were used as antigens. The first efforts to make anti-ovine AANAT by Joan Weller provided evidence that there was a close association between AANAT activity and protein. Jonathan Gastel extended this into studies in the rat, and made the very important discovery that the abundance of AANAT protein is regulated by proteasomal proteolysis, which explains how pineal AANAT activity rapidly disappears following exposure to light, at night (11). It is suspected that proteolysis involves a highly conserved lysine in the N-terminal region of the protein. It will be of interest to determine if this mechanism is conserved and explains photic suppression of AANAT activity in all vertebrates.

Kinetic analysis of AANAT was made possible by the preparation of a GST-expression construct by Jonathan Gastel. Using this construct, Phil Cole has been able to study the mechanism of acetylation and to predict that acetyl transfer involves the formation of an intermediate quarternary tryptamine-AcCoA complex (10). This is being supported by the results of structural analysis of AANAT by Alison Hickman and Fred Dyda (64). The structural work has used a highly soluble form of ovine AANAT to obtain crystals for X-ray crystallography. This reflects an important collaboration with Servier and their intention to develop AANAT-targeted drugs.

A striking outcome of the molecular analysis of AANAT regulation is the realization that there are distinct species-to-species differences in the mechanisms through which AANAT is regulated in vertebrates. However, it is clear that these represent "variations on a theme" and that in nearly all cases these mechanisms serve to insure that melatonin synthesis is only elevated at night in the dark. The apparent differences

seem to serve to fine-tune the system so that it functions to optimize survival and behavior as required for each species.

4. THE FUTURE OF AANAT

In what directions will AANAT research go? It is reasonable to predict that continued investigations of AANAT will lead to a better understanding of clock control of gene expression in vertebrates; this should come form studies on the chicken and pike. Studies on rat AANAT promoters will shed light on the molecular basis of control of tissue specific expression of genes in the pineal gland and retina. Analysis of AANAT degradation should lead to a detailed understanding of this process in the pineal gland and will also reveal how proteolysis is regulated in the nervous system. Pharmacology studies are expected to lead to the identification of agents that modify AANAT activity in the pineal gland and elsewhere, with the possible evolution of such agents into AANAT-directed drugs that influence sleep and behavior. I expect that studies on the structure of AANAT will enhance our understanding of fundamental elements of enzyme action and will also provide a description of three dimensional surfaces that will be important in designing drugs and in understanding protein-protein interactions. There is good reason to suspect that the use of AANAT as a marker will enhance our understanding of embryological differentiation. I'm confident that these predictions will be realized. I am equally confident that there are lots of eager, imaginative and creative young scientists unknown to me who will make spectacular and surprising advances with AANAT in areas I haven't touched. Seeing how this develops will be interesting.

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